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ACCELERATED COMMUNICATION

Selective association of protein molecules followed by mass spectrometry

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Abstract

Nanoflow electrospray mass spectrometry was used to monitor the formation of protein heterodimers of HU proteins from *Bacillus stearothermophilus* and *Bacillus subtilis*. This has enabled us to analyze both thermodynamic and kinetic features associated with the dissociation of homodimeric HU proteins. The results obtained correlate well with the kinetics of the protein dissociation process and the free energy difference between homo- and heterodimeric species anticipated from other studies. We suggest that this approach will have general applicability in studying protein association and dissociation under near-equilibrium conditions and will be relevant to a wide range of biological systems.

Keywords: HU proteins; nanoflow electrospray mass spectrometry; protein interactions

We have applied nanoflow electrospray mass spectrometry (nano-ES MS) (Wilm & Mann, 1994, 1996) to characterize dimers of the type II DNA-binding proteins (Drlica & Rouvière-Yaniv, 1987; Rice, 1997) from different bacterial species. In their functional states, the HU proteins from *Bacillus stearothermophilus* (HBst) and from *Bacillus subtilis* (HBsu) are homodimers of intertwined polypeptides containing 90 and 92 amino acid residues, respectively. Both the crystal and solution structures have been determined for HBst (White et al., 1989; Vis et al., 1995). HBsu differs in sequence from HBst at only 12 of the amino acid positions, and by a two amino acid extension at the C-terminus. Nevertheless, the stabilities of the two proteins vary considerably under some conditions (Wilson et al., 1990; Kawamura et al., 1996). The thermostability of HBst is much higher than that of HBsu in aqueous solution in the absence of salt, although at high ionic strength the denaturation temperatures of both proteins become more similar (Welfle et al., 1992). Their structural similarity permits the formation of heterodimers, but preferential formation of homodimers occurs at high ionic strength where the protein stabilities are highest. This enables us to probe thermodynamic and kinetic features of the dissociation process. We propose that this approach has general use in investigating association of biomolecules in solution.

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Results and discussion

ES MS produces multiply charged gas-phase ions from protein molecules in solution in which noncovalent features of the structure are maintained (Przybylski & Glocker, 1996). It therefore provides a powerful method for the measurement of the populations of different conformational states within an ensemble of protein molecules. The mass spectra of a 1:1 mixture of HBst and HBsu at 350 mM ammonium acetate, recorded rapidly after mixing at 20 °C, are shown in Figure 1. At low cone voltages, well-defined charge states of the two proteins are observed (Fig. 1A). The mass/charge-separation of consecutive charge states indicates that they are both dimeric species, having charges of 8+, 9+, and 10+. The dimers differ significantly in mass (19,440 ± 3 Da for HBst and 19,776 ± 2 Da for HBsu) and hence the spectrum of a mixture gives two well-resolved series of charge states. At high cone voltages (Fig. 1B), however, the noncovalent interactions of dimers in the gas phase are disrupted (Nettleton et al., 1998) and charge states corresponding only to monomers are observed. At intermediate cone voltages, the signals of both monomers and homodimers are observed (Fig. 1C). When the solution containing both proteins was heated to 80 °C for 30 min and a mass spectrum recorded after cooling to 20 °C at an intermediate cone voltage, a spectrum showing three rather than two signals for all dimeric charge states was observed (Fig. 1D). This indicates that heterodimers as well as homodimers are present in the solution. This spectrum enables us to analyze the distribution of the different species in solution.

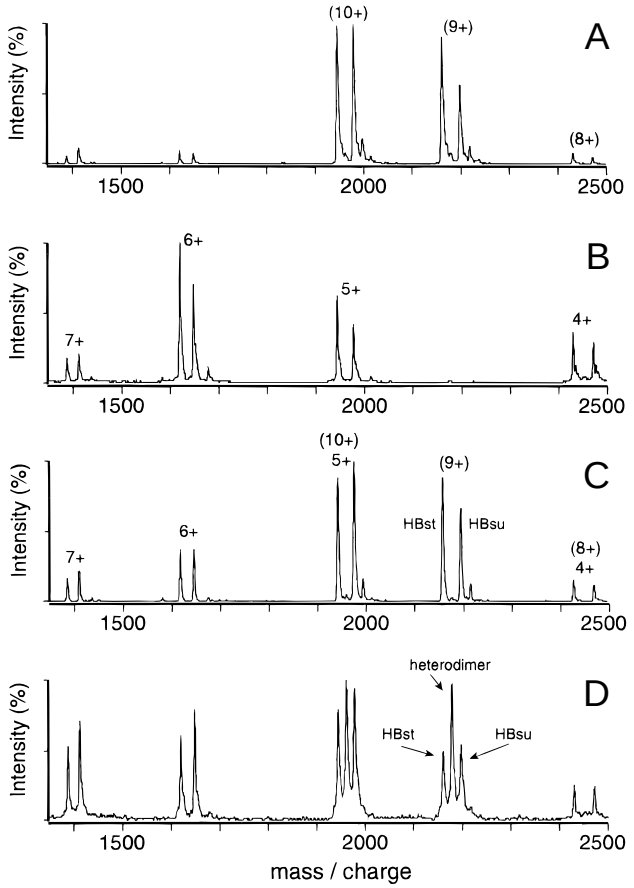

Fig. 1. Nano-ES mass spectra of a 1:1 mixture of HBst and HBsu at different cone voltages. (**A**) Charge states of both homodimers at a low cone voltage of 55 V; (**B**) charge states of both monomers at a high cone voltage of 110 V. Both monomer and dimer charge states were observed at an intermediate cone voltage of 80 V, (**C**) before and (**D**) after heating to 80 °C for 30 min and cooling down to 20 °C. The proteins were dissolved in aqueous solution containing 350 mM ammonium acetate adjusted to pH = 5.0 using formic acid. The labels on the peaks $n+$ indicate the number of excess positive charges n on the protein ion. Labels with and without parenthesis correspond to charge states of dimers and monomers, respectively.

The mass derived from the central peak of the 9+ and 10+ charge states is 19,608 ± 2 Da, which corresponds exactly to the mass expected for the heterodimer of HBst and HBsu. Since the three peaks identified for the 9+ dimeric ions do not overlap with charge states from monomers, the fractional population of heterodimers, F_{het}, can be obtained by dividing the intensity of the central peak by the total intensity of this charge state. The value of F_{het} in Figure 1D is 0.49 ± 0.02, implying that the dimers are almost completely randomized after heating the mixture. (Note: Assuming that the MS response for the 9+ charge states is similar for homo- and heterodimeric species, the predicted value of F_{het} is 0.5, when both the structure and stability of the two proteins are the same, and 0.0 when no interconversion between these proteins occurs.) An analysis of nano-ES mass spectra recorded at different cone voltages with the sample containing the three dimer species shows that, although the fraction of HU dimer molecules decreases almost linearly on increasing the cone voltage in the range from 50–110 V, the value of F_{het} remains approximately the same at the different cone voltages.

A series of nano-ES mass spectra was subsequently recorded at different time points after mixing solutions at 20 °C containing the two homodimers at different ammonium acetate concentrations. Figure 2 shows the fractional population of heterodimers obtained from these spectra, which can be seen to increase very slowly with time. The slow interconversion of the dimeric species is in accord with expectations based on a simple dissociation–association model.

Intuitively, it is reasonable to assume that the formation of a heterodimer involves the dissociation of different homodimers into monomers, which may be partially folded (Vis et al., 1998), followed by the re-assembly of two different monomers. Due to the high stability of dimer at 500 mM salt, expressed by an association constant of 10^9 M (Welfle et al., 1993), the population of monomeric molecules will be very low. This explains the extremely slow kinetics for establishing equilibrium fractions of homo- and heterodimers of HU. In fact, assuming the simplest model for the process of dissociation and re-association, with the concentration of a monomeric protein used here $[HU] = 5.0 \cdot 10^{-5}$ M and the buildup rate of heterodimers $k = 5.8 \cdot 10^{-6}$ s^{-1} (Fig. 2), the predicted rate of refolding of HU dimers would be of the order of 1.0 s^{-1}. This value is consistent with an estimate of the experimental rate of refolding obtained using stopped-flow fluorescence.

Both the kinetics of establishing the equilibrium distribution and the values of F_{het} at equilibrium decrease as the ionic strength of the solution is increased. At 500 mM salt, F_{het} at equilibrium is only 0.14 ± 0.02, indicating that at high ionic strength the propensity to form heterodimers is very much reduced. This result is confirmed by an experiment in which establishing of the equilibrium populations of dimers was monitored following randomization of the populations by thermal unfolding (Fig. 3). The value of $\Delta G = 5.44$ kJ mol^{-1} (at 500 mM salt) calculated for this distribution is in reasonable agreement with the difference between the

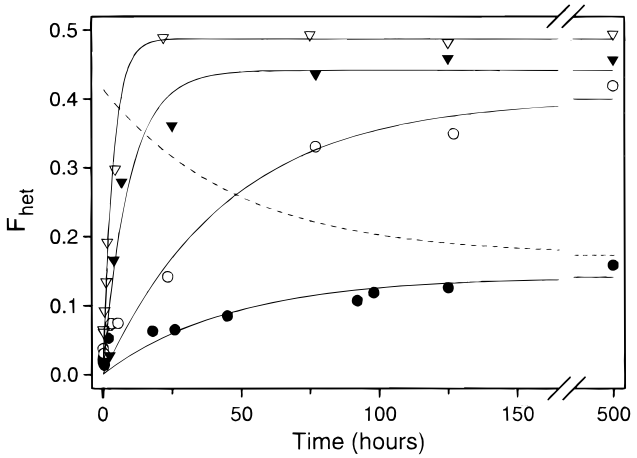

Fig. 2. Time dependence of the formation of heterodimers of HBst and HBsu at different ammonium acetate concentrations. All curves fit to a single-exponential function, parameterized with a rate constant k and the fractional population F_{het} at equilibrium. Ammonium acetate concentrations were: ▽, 135 mM ($k = 0.260 \pm 0.030$ h^{-1}, $\{F_{het}\}^{eq} = 0.487 \pm 0.014$); ▼, 180 mM ($k = 0.103 \pm 0.022$ h^{-1}, $\{F_{het}\}^{eq} = 0.442 \pm 0.024$); ○, 290 mM ($k = 0.022 \pm 0.006$ h^{-1}, $\{F_{het}\}^{eq} = 0.399 \pm 0.030$), and ●, 500 mM ($k = 0.021 \pm 0.007$ h^{-1}, $\{F_{het}\}^{eq} = 0.141 \pm 0.016$). The dashed curve shows the time-decay of F_{het} after heating the mixture containing 500 mM salt to 80 °C and cooling down to 20 °C (Fig. 3). The values of $k = 0.020 \pm 0.007$ h^{-1} and $\{F_{het}\}^{eq} = 0.172 \pm 0.016$ are very similar to the values obtained for the formation of heterodimers at the same salt concentration.

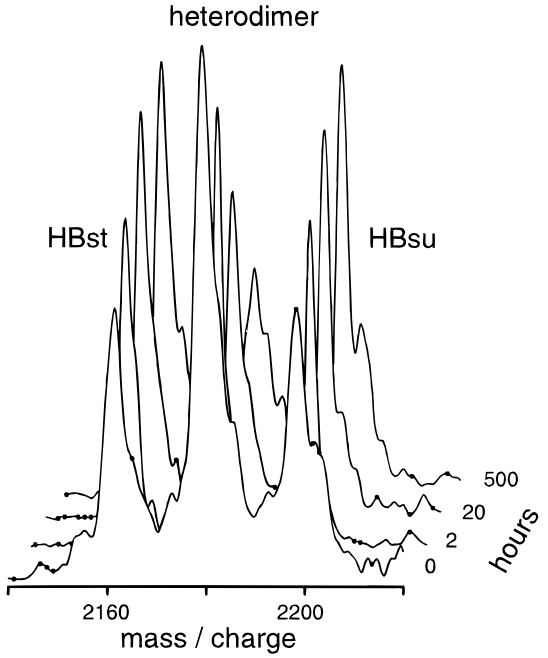

Fig. 3. Mass spectra showing the time dependence of the 9+ charge state assigned to dimers formed from HBst and HBsu. Solutions of HBst and HBsu containing 500 mM ammonium acetate were mixed, heated to 80 °C to unfold the proteins and cooled down to 20 °C. Mass spectra were obtained at different time points using a cone voltage of 80 V. The fractional populations of the dimers are initially random, but when equilibration takes place those of both homodimers increases, while that of the heterodimer decreases.

free-energy change of association, 6.50 kJ mol^{-1}, of wild-type HBst, and of one of its less stable mutants, HBst-T13A/G15E, in which the substituted residues are those found at the same position in HBsu (Kawamura et al., 1996).

The results discussed in this paper show that it is possible to analyze both thermodynamic and kinetic features associated with the dissociation of proteins in solution using nano-ES MS. We suggest that this approach is generally applicable to the study of protein association and dissociation under near-equilibrium conditions.

Materials and methods

Sample preparation

Previously published methods for cloning, expression, and purification of the proteins HBst (Padas et al., 1992) and HBsu (Groch et al., 1992) were employed.

Free energy calculation

The equilibrium thermodynamics of the interconversion of two homodimeric states present in equimolar quantities and their heterodimer state can be described by a constant $K_{eq} = (2 * \{F_{het}/(1 - F_{het})\})^2$. Using this expression, the free energy difference can be calculated according to $\Delta G = -RT \ln K_{eq}$ (R = 8.314 J K^{-1} mol^{-1} is the molar gas constant, and T is the temperature (K)).

Mass spectrometry

All mass spectra were acquired over the m/z range 1,300–2,500 Da/e on a Platform II mass spectrometer (Micromass, Manchester, UK) at 20 °C without source heating and in the absence of organic co-solvents. The capillary voltage was 1.4 kV. 3 μL of 0.5 mg/mL protein solution were introduced via the nanoflow electrospray interface using gold-plated needles, which were prepared in-house from borosilicate glass capillaries of external diameter 0.5 mm on a Model P-97 Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, California) with a final gold coating applied with an SEM coating system (Polaron, Sittingborne, UK).

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